

## Isolation and Identification of a Variant of Bluetongue Virus Serotype 11 from a Ram in a Bluetongue Outbreak in Western Texas

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Received 26 December 1990/Accepted 22 April 1991

**A field strain of bluetongue virus was isolated from a blood sample of a ram during an outbreak of bluetongue in November 1985 in western Texas. In this bluetongue outbreak at least 25 of the 2,000 sheep were infected. Isolation was made by intravenous inoculation of 11-day-old embryonated chicken eggs. The serotype was identified as serotype 11 by serum neutralization tests. The genomic pattern on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the new isolate is similar to that of bluetongue virus prototype 11. Comparisons were also made with proteins labeled in vivo with [<sup>3</sup>H]leucine and separated by SDS-PAGE. We conclude that this virus belongs to serotype 11, with slight differences in both genome and protein electrophoretic patterns.**

Bluetongue virus (BTV), a member of the *Reoviridae* family, *Orbivirus* genus, is arthropod borne and replicates in both *Culicoides* vectors and ruminant hosts. BTV causes a disease characterized by fever, inflammation, and congestion of oral and nasal mucous membranes. Sheep, in particular, may suffer from a severe and often fatal form of the disease; in cattle and goats, bluetongue is generally subclinical, and virus can frequently be isolated from apparently healthy animals (2). Infection during early gestation may result in congenital malformations or death of the developing fetus (17, 21) or lead to persistently infected carriers (15, 16). Serotypes 2, 10, 11, 13, and 17 have been recovered from animals in the United States (1, 6). BTV possesses a genome that is composed of 10 segments of double-stranded RNA (28). In the virus particle the double-stranded RNA is surrounded by a double-capsid shell, the outer capsid consisting of two proteins (proteins 2 and 5) and the inner core consisting of five proteins including proteins 1, 3, 4, 6, and 7 (18). The double-stranded RNA segments can be separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) into distinct electrophoretic patterns or electropherotypes. Electrophoretic polymorphisms have been reported for different serotypes (8, 10, 13) and for different strains within a single serotype (13, 23). The genetic diversity of BTV has been analyzed by PAGE (8, 13, 23, 27) and two-dimensional gel electrophoresis of RNase T<sub>1</sub>-digested RNA (26, 29). These techniques have the advantage of providing information on all 10 genome segments, each coding for unique proteins (7, 27), whereas serotyping assays identify primarily external proteins (11, 12).

The isolation and identification of a BTV serotype 11 from the blood of a sheep during a BTV outbreak in western Texas in the fall of 1985 is presented in this communication. During the outbreak, at least 25 sheep in a herd of 2,000 were clinically infected with bluetongue. The isolate, called Ram virus, was isolated from a heparinized blood sample from a ram having bluetongue symptoms. The isolation was made by intravascular inoculation of 11-day-old embryonated chicken eggs (5). All egg embryos injected with the isolate

died within 7 days after inoculation. The egg embryos that died within 24 h were not included. The embryos were homogenized, and the homogenates were inoculated onto Vero cells. Once cytopathic effects were observed, the Ram virus was harvested and stored at -70°C. The Ram virus caused quicker death of embryonated chicken eggs (within 7 days) and produced cytopathic effects faster (within 2 days) and a higher titer in BHK-21 cells (100 50% tissue culture infective doses higher) than U.S. prototype BTV 11. The virus was plaque purified three times on Vero cells. The U.S. BTV prototype strains used and collection sites were the following: serotype 10, strain 8, California; serotype 11, Station strain, Texas; serotype 13, strain 67-41B, Idaho; serotype 17, strain 62-45S, Wyoming; and serotype 2, strain Ona A, Florida (4). All viruses were propagated in BHK-21 cells before electropherotype determinations.

Viral RNA was extracted from BTV-infected BHK-21 cells by using phenol in sodium acetate buffer, pH 5.0 (4). The genomic electropherotype of each serotype was determined by SDS-PAGE on 1.5-mm 10% gels with 5% stacking gels (4, 14). Gels were washed with ethanol and acetic acid, stained with silver for 1.5 h, rinsed, and fixed for 10 min in 5% acetic acid. After a final rinse, the gels were stored in water. When compared with all five U.S. BTV prototypes, the genomic SDS-PAGE electropherotype of Ram virus was the closest to that of prototype BTV 11 (Fig. 1, comparison of BTV 10, BTV 11, and the Ram virus). An extra band appears in front of segment 4, and segments 8 and 9 of the Ram virus seem to migrate together (Fig. 1). Segment 10 of the Ram virus ran farther than that of BTV 11 on SDS-PAGE (Fig. 1). Point mutation, genetic shift, or reassortment might have caused the slightly different patterns of genome segments 4, 8, 9, and 10 on SDS-PAGE between BTV prototype 11 and the Ram virus. The extra band in front of segment 4 of the Ram virus may be due to a genetically heterogeneous but well adapted population of BTV serotype 11 (6).

Cultures of 10<sup>6</sup> BHK cells were infected with 1 ml of 10<sup>6</sup> 50% tissue culture infective doses of virus for viral protein labeling. After 18 h of incubation, the infected monolayers were washed with Eagle minimum essential medium lacking leucine. The medium was then replaced with the same medium containing 100 µCi of [<sup>3</sup>H]leucine. After 3 to 4 h of

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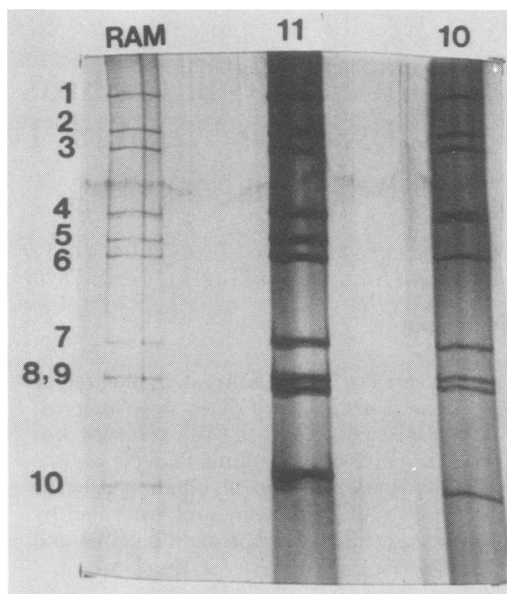


FIG. 1. Genomic electropherotypes of the Ram virus and BTV prototypes 10 and 11.

incubation at 37°C, the medium was removed and the cells were washed and then lysed by the addition of loading buffer (4, 12, 14). Labeled viral proteins were separated by SDS-PAGE as described above and identified by autoradiography. Polypeptide electropherotypes of BHK-21 cell protein extract, BTV 11, and Ram viral proteins were compared. Proteins 4, NS-1, 6, and 7 of the Ram virus have slightly different mobilities than those from BTV 11 on the SDS-PAGE (Fig. 2). Protein NS-2 of the Ram virus was not clearly visible on SDS-PAGE. Both proteins 2 and 5 of the Ram virus and BTV 11, which are part of the outer capsid, migrated at a similar rate (i.e., were of similar molecular weight).

Serum neutralization tests were conducted in Vero cell monolayers. Vero cells were grown in Eagle minimum essential medium supplemented with 10% fetal bovine serum (FBS). All the sera tested were inactivated by incubation at 56°C for 30 min. Twofold serial dilutions of anti-BTV 2, 10, 11, 13, and 17 sera were incubated with an equal volume of 100 50% tissue culture infective doses of Ram virus per ml at 37°C for 60 min. The serum-virus mixtures were then inoculated onto Vero cell monolayers and incubated at 37°C for another 60 min before addition of Eagle minimum essential medium with 2% FBS. Each dilution was tested in triplicate and checked for cytopathic effects every day up to day 6 postinoculation. Ram virus was neutralized by anti-BTV 11 serum up to a dilution of 1:128, whereas the prototype strain of BTV 11 was neutralized by a dilution of anti-BTV 11 serum of 1:64. Ram virus was not neutralized by the other anti-U.S. prototype sera.

It has been shown that BTV strains of a single serotype, exhibiting more than 1 electropherotype, may coexist within a single animal or herd (4, 28). In addition, predominant electropherotypes have been shown to change during the course of a single bluetongue outbreak (4). Samal et al. (22) have identified sheep as a natural host within which reassortment of BTV genome segments can occur. Epizootiological studies in the western United States indicate that up to one-third of all BTV-infected herds had more than one

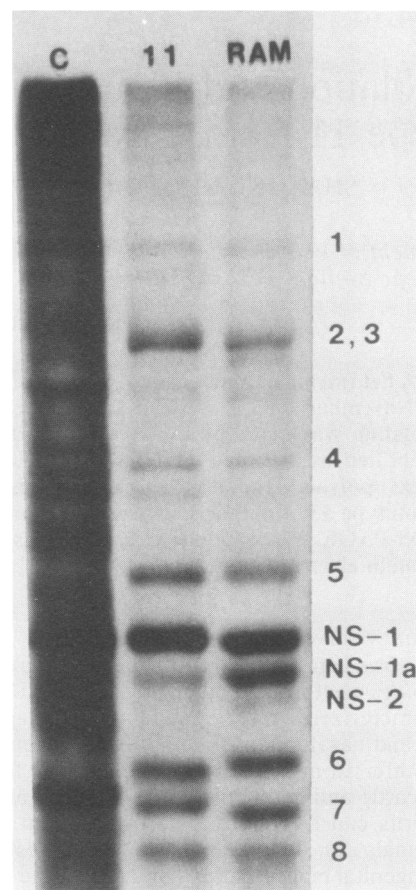


FIG. 2. Protein electropherotypes of the Ram virus, BTV prototype 11, and BHK-21 cell control (C).

serotype on the premises (20). Moreover, multiple serotypes have been recovered from individual infected sheep and cattle (19, 24). The potential for intertypic genetic interactions in mixedly infected hosts is unknown, but results from in vitro mixed infections suggest that reassortment of genome segments should be expected to occur at a high frequency (12). The notion that reassortment among BTV serotypes occurs in nature is supported by evidence for naturally occurring reassortment between BTV serotypes 10 and 11 (26) and within serotype 11 (25). When inoculated simultaneously into sheep with serotypes 10 and 17, the serotype 10 parental electropherotype predominated among the progeny and contributed the majority of the genome segments to reassortant viruses (22). The existence of these naturally occurring reassortments among BTV serotypes has prevented the use of polyvalent vaccines in the United States, although such vaccines have been used in South Africa and Israel (3, 9).

According to the coding assignments for the genome segments of BTV serotype 1, genome segments 1, 2, 3, 4, 5, and 7 code for proteins 1, 2, 3, 4, 5, and 7, respectively, genome segment 6 codes for proteins NS1 and NS1a, segment 8 codes for protein NS2, segment 9 codes for protein 6, and segment 10 codes for proteins 8 and 8a (18). This study of BTV 11 and the Ram virus shows those genome segments having similar mobilities; coding for proteins with similar rates of migration are segments 1, 2, 3, and 5 (proteins 1, 2,



3, and 5, respectively). Segments 6 and 7 of BTV 11 and the Ram virus migrate at a similar rate but code for proteins which migrate differently (NS-1 and protein 7, respectively). Segments 4, 8, and 9 of BTV 11 and the Ram virus have detectable differences in electrophoretic mobilities, and the corresponding proteins, 4, NS-2 (at a lower amount in BTV 11 than in the Ram virus), and 6, respectively, also have differences in migration. Segment 10 in both BTV 11 and Ram virus migrated differently, but the proteins (protein 8) they encode migrated similarly. This segment is possibly a result of reassortment between two different serotypes of BTV, such as BTV 10 and BTV 11 (Fig. 1).

The serum neutralization data indicate that Ram virus belongs to serotype 11. The polypeptide electrophoresis shows that proteins 1, 2, 3, 5, and 8 of BTV 11 and Ram virus seem to have the same molecular weight. Among these five proteins, protein 2, which is the target of neutralizing antibodies and, thus, the serotype-specific protein, is coded by genome segment 2 (12). This also suggests that BTV 11 and Ram virus are in the same serogroup. We conclude that although the Ram virus has the same serotype as BTV 11, the general genetic data indicate that it is very different from BTV prototype 11. Electrophoretic polymorphisms have been previously reported for different serotypes (8, 10, 13) and for different strains within a single serotype (13, 23). These polymorphisms have provided useful biochemical markers for the analysis of genetic reassortment in *in vitro* and *in vivo* crosses of BTV (12, 22). In order to understand the genetics and epidemiology of BTV and, further, to produce a vaccine for it, it is necessary to characterize BTV field isolates not only by serotyping but also by electropherotyping.

#### REFERENCES

1. Barber, T. L. 1979. Temporal appearance, geographic distribution and species of origin of bluetongue virus serotypes in the United States. *Am. J. Vet. Res.* **40**:1654-1656.
2. Barber, T. L., and E. W. Collisson. 1985. Epidemiologic implications of the genetic variations of bluetongue virus, p. 597-606. *In* T. L. Barber and M. M. Jochim (ed.), *Bluetongue and related orbiviruses*. Alan R. Liss, Inc., New York.
3. Barzilai, E., and A. Shimshony. 1985. Bluetongue: virological and epidemiological observations in Israel, p. 545-553. *In* T. L. Barber and M. M. Jochim (ed.), *Bluetongue and related orbiviruses*. Alan R. Liss, Inc., New York.
4. Collisson, E. W., T. L. Barber, E. P. J. Gibbs, and E. C. Greiner. 1985. Two electropherotypes of bluetongue virus serotype 2 from naturally infected calves. *J. Gen. Virol.* **66**:1279-1286.
5. Foster, N. M., and A. J. Luedke. 1968. Direct assay for bluetongue virus by intravascular inoculation of embryonating chicken eggs. *Am. J. Vet. Res.* **29**:749-753.
6. Gibbs, E. P. J., E. C. Greiner, W. P. Taylor, T. L. Barber, J. A. House, and J. E. Pearson. 1983. Isolation of bluetongue virus serotype 2 from cattle in Florida: a serotype of bluetongue virus hitherto unrecognized in the Western Hemisphere. *Am. J. Vet. Res.* **44**:2226-2228.
7. Gorman, B. M. 1979. Variation in orbiviruses. *J. Gen. Virol.* **44**:1-15.
8. Gorman, B. M., J. Taylor, P. J. Walker, W. L. Davison, and F. Brown. 1981. Comparison of bluetongue type 20 with certain viruses of the bluetongue and Eubanangae serological groups of orbiviruses. *J. Gen. Virol.* **57**:251-261.
9. Howell, P. G., and D. W. Verwoerd. 1971. Bluetongue virus. *Virol. Monogr.* **9**:35-47.
10. Huismans, H., and C. W. Bremer. 1981. A comparison of an Australian bluetongue virus isolate (CSIRO 19) with other bluetongue virus serotypes by cross-hybridization and cross-immune precipitation. *Onderstepoort J. Vet. Res.* **48**:59-67.
11. Huismans, H., and B. J. Erasmus. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J. Vet. Res.* **48**:51-58.
12. Kahlon, J., K. Sugiyama, and P. Roy. 1983. Molecular basis of bluetongue virus neutralization. *J. Virol.* **48**:627-632.
13. Knudson, D. L., W. K. Butterfield, R. E. Shope, T. E. Walton, and C. H. Campbell. 1982. Electrophoretic comparison of the genomes of North America bluetongue viruses, one Australian bluetongue virus and three other related orbiviruses. *Vet. Microbiol.* **7**:285-293.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
15. Luedke, A. J., M. M. Jochim, and T. L. Barber. 1982. Serologic and virologic responses of a Hereford bull persistently infected with bluetongue virus for eleven years. *Proc. Annu. Meet. Am. Assoc. Vet. Lab. Diagn.* **25**:115-134.
16. Luedke, A. J., M. M. Jochim, and R. H. Jones. 1977. Bluetongue in cattle: effects of *Culicoides variipennis*-transmitted bluetongue virus on pregnant heifers and their calves. *Am. J. Vet. Res.* **38**:1687-1694.
17. MacLachlan, N. J., and B. I. Osburn. 1983. Bluetongue virus-induced hydranencephaly in cattle. *Vet. Pathol.* **20**:563-573.
18. Mertern, P. P. C., F. Brown, and D. V. Sangar. 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology* **135**:207-217.
19. Oberst, R. D., K. R. E. Squire, J. L. Stott, R. Y. Chuang, and B. I. Osburn. 1985. The coexistence of multiple bluetongue virus electropherotypes in individual cattle during natural infection. *J. Gen. Virol.* **66**:1901-1909.
20. Osburn, B. E., B. McGowan, B. Heron, E. Loomis, R. Bushnell, J. Stott, and W. Utterback. 1981. Epizootiologic study of bluetongue: virologic and serologic results. *Am. J. Vet. Res.* **42**:884-887.
21. Osburn, B. I., A. M. Silverstein, R. A. Prendergast, R. T. Johnson, and C. J. Parshall, Jr. 1971. Experimental viral-induced congenital encephalopathies. *Lab. Invest.* **25**:197-205.
22. Samal, S. K., C. W. Livingston, Jr., S. McConnell, and R. F. Ramig. 1987. Analysis of mixed infection of sheep with bluetongue virus serotype 10 and 17: evidence for genetic reassortment in the vertebrate host. *J. Virol.* **61**:1086-1091.
23. Squire, K. R. E., B. I. Osburn, R. Y. Chuang, and R. H. Doi. 1983. A survey of electropherotype relationships of bluetongue virus isolates from the Western United States. *J. Gen. Virol.* **64**:2103-2115.
24. Stott, J. L., B. I. Osburn, and T. L. Barber. 1982. Recovery of dual serotypes of bluetongue virus from infected sheep and cattle. *Vet. Microbiol.* **7**:197-207.
25. Sugiyama, K., D. H. Bishop, and P. Roy. 1982. Analyses of the genome of bluetongue viruses recovered from different states of the United States at different times. *Am. J. Epidemiol.* **115**:332-347.
26. Sugiyama, K., D. H. L. Bishop, and P. Roy. 1981. Analyses of the genomes of bluetongue viruses recovered in the United States. I. Oligonucleotide fingerprint studies that indicate the existence of naturally occurring reassortant BTV isolates. *Virology* **114**:210-217.
27. Verwoerd, D. W., H. Huismans, and B. J. Erasmus. 1979. Orbiviruses, p. 285-345. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 14. Plenum Press, New York.
28. Verwoerd, D. W., H. Louw, and R. A. Oellerman. 1970. Characterization of bluetongue virus ribonucleic acid. *J. Virol.* **5**:1-7.
29. Walker, P. J., J. N. Mansbridge, and B. M. Gorman. 1980. Genetic analysis of orbiviruses by using RNase T1 oligonucleotide fingerprints. *J. Virol.* **34**:583-591.